

S/N 10/594100

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Geng et al.	Examiner:	Maier, Leigh C.
Serial No.:	10/594100	Group Art Unit:	1623
Filed:	June 29, 2007	Docket No.:	09458.1045USWO
Title:	ALGIN OLIGOSACCHARIDES AND THE DERIVATIVES THEREOF AS WELL AS THE MANUFACTURE AND THE USE OF THE SAME		

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DECLARATION UNDER 37 CFR §1.132

Mail Stop Amendment  
Commissioner for Patents  
P.O. Box 1450  
Alexandria, Virginia 22313-1450

Dear Sir:

I, Meiyu Geng, pH.D, hereby declare as follows:

1. I obtained my bachelor degree in Medicine in 1986 and master degree in pharmacology in 1989 from Shandong Medical University (Now Shandong University). I got my Ph.D in Pharmacy from Tokyo University in 1996.

2. I have worked in Marine Drug and Food Institute of Ocean University of China since 1989. I have started from the discovery to the development of carbohydrate-based drugs covering a descriptive phenomenon into molecular understanding in Alzheimer's treatment to cancer therapy in the past decade. Major interests are mainly focused on the research and development of targeted molecular agents in particular A $\beta$ -targeting inhibitors, and deciphering of the possible molecular mechanisms in signal transduction. Currently, I am also focusing on characterizing genomics-based new targets and investigating the impact of biomarkers in AD progression and therapy response as well.

Based on the established glyco-microarray technique for high-throughput and micro-scale screening of biologically active marine-derived oligosaccharides, I have found a series of potential oligosaccharide-based drug candidates, including anti-AD drug candidate oligomannurate and heparanase inhibitor JG3. During the past decades, more than 60 papers have been published in peer-reviewed journals and over 10 patents have been filed and 5 patents have been authorized.

I found that oligomannurate, a novel marine-derived oligosaccharide, inhibits the entire fibril-forming process by stabilizing A $\beta$  in an  $\alpha$ -helical state, by driving disassembled fibrils into non-toxic conformers both in vitro and in a transgenic mouse model. Notably, this efficacy occurs via the binding capacity of oligomannurate for N-terminus and  $\beta$ -hairpin species at different stages by simultaneously targeting SNK and HHQK domains on A $\beta$  peptide. These features, together with good oral bioavailability, blood-brain barrier

accessibility, and favorable safety and tolerability in newly completed Phase I clinical trials, make oligomannurate both a prophylactic and therapeutic drug candidate for AD therapy. Now, oligomannurate is under phase II clinical trial in China.

Inhibitors of tumor angiogenesis and metastasis are increasingly emerging as promising agents for cancer therapy. Recently, heparanase inhibitors have offered a new avenue for such work because heparanase is thought to be critically involved in the metastatic and angiogenic potentials of tumor cells. I found that oligomannurate sulfate (JG3), a novel marine-derived oligosaccharide, acts as a heparanase inhibitor to inhibit tumor angiogenesis and metastasis both *in vitro* and *in vivo* by combating heparanase activity via binding to the KKDC and QPLK domains of the heparanase molecule, making JG3 a promising candidate agent for cancer therapy.

In addition, I have also discovered another sulfated polymannuroguluronate (SPMG), extracted from brown algae followed by chemical modification, inhibited HIV replication via its binding to the V3 region of the capsid glycoprotein molecule of the virus, gp120, therefore interrupting the binding of V3 region to CXCR4 and CCR5 (both are the co-receptors to CD4 molecule) and further preventing the entry of HIV into the host cells.

3. I am one of the inventors for the invention described in US Patent Application No. 10/594100 and am familiar with the subject matter thereof.

Alzheimer's disease (AD) is a devastating neurological disorder that affects more than 37 million people worldwide. The economic burden of AD is massive. Currently approved drugs for AD ameliorate symptoms for a short time by boosting levels of neurotransmitters, but do not alter the general progression or outcome of the disease.

Intense efforts have been devoted to finding disease-modifying therapies that target the underlying AD pathogenic molecules. Of these,  $\beta$ -amyloid peptide ( $A\beta$ ), a 39-43 residue cleavage product of amyloid precursor protein, is the main component of senile plaques of AD, constitutes the focus of current interest.

Since amyloid fibril formation is a multi-stage process involving different  $A\beta$  species at different stages, an exciting current anti-AD strategy is to challenge mechanism-based multi-targeting agents. To this end, inhibitors should be broadly active across multiple stages of fibrillation. Such ideal agents are thus anticipated to stabilize  $A\beta$  in monomeric state that are unable to further assemble, favor the disassembly of high molecular-weight oligomers and fibrillar deposits in non-toxic conformers, and encourage its clearance through normal pathways by maintaining the  $A\beta$  in a monomer state.

With the availability of various synthetic  $A\beta$  species and a marine-derived carbohydrate library in our lab, a comprehensive screening program was undertaken. Oligomannurate, an acidic oligosaccharide obtained from degradation and subsequent chemical modification, stood out as a full inhibitor of  $\beta$ -amyloid cascades by binding to Ser26-to-Lys28 (SNK) residues and simultaneously to the HHQK motif in  $A\beta$  peptide. oligomannurate arrests fibril formation by stabilizing  $A\beta$  in an  $\alpha$ -helix, and destabilizes fibrils into non-toxic conformers both *in vitro* and in a transgenic mouse model. The applied patent with No. 10/594100 generated from this product oligomannurate.

4. Under my direction, the following experiments were conducted for the purposes of showing that the compound obtained in US Patent Application No.10/594100 (named alginate

oligosaccharide derivatives) has the preventive effects against Alzheimer's disease (AD) and type 2 diabetes.

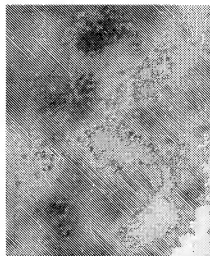
### **Experiment 1**

#### **Alginate oligosaccharide derivatives inhibit A $\beta$ aggregation**

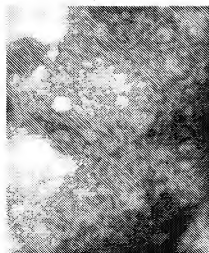
The aggregation of the amyloid- $\beta$  (A $\beta$ ) peptide into toxic, prefibrillar oligomers is considered to be the key pathogenic event for AD initiation and progression. Thus, inhibiting A $\beta$  aggregation is able to prevent the initiation of the AD. Thus we first observed the effects of alginate oligosaccharide derivatives on A $\beta$ 1-42 aggregation with transmission electron microscopy (TEM). After 96 h incubation, A $\beta$ 1-42 formed fibrils of classic morphology (Fig.1). Incubation of A $\beta$ 1-42 with heparin resulted in an even more densely packed and lateral aggregated A $\beta$  fibril structure. Alginate oligosaccharide derivatives prevented fibril formation of both A $\beta$ 1-42 alone and heparin-bound A $\beta$ 1-42. Occasionally, very sparse irregular dots were observed.



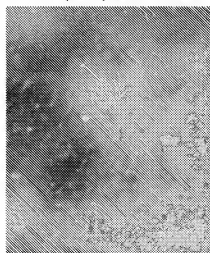
A $\beta$ 1-42



A $\beta$ 1-42+alginate oligosaccharide derivatives



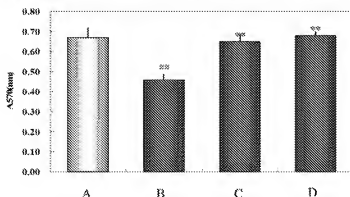
A $\beta$ 1-42+heparin



A $\beta$ 1-42+heparin+alginate oligosaccharide derivatives

**Figure 1** Effects of alginate oligosaccharide derivatives on A $\beta$ 1-42 fibril formation as observed by TEM. Monomeric A $\beta$ 1-42 was incubated alone or with alginate oligosaccharide derivatives (1:20) or heparin (2:1), or with both alginate oligosaccharide derivatives (1:20) and heparin (2:1), at 37°C for 96 h and observed with TEM. Scale bars are 100 nm. Data are representative of at least three independent experiments.

To further confirm the inhibiting effect of alginate oligosaccharide derivatives on A $\beta$  aggregation, we observed the neuronal toxicity of alginate oligosaccharide derivatives treated A $\beta$ 1-42 using SRB assay. In SH-SY5Y cells, treatment with 4  $\mu$ M aggregated A $\beta$ 1-42 resulted in significant neuronal toxicity, compared with control. Alginate oligosaccharide derivatives treated A $\beta$ 1-42 induced almost no neuronal toxicity (Fig. 2).



**Figure 2** Neurotoxicity of alginate oligosaccharide derivatives treated A $\beta$ 1-42. Cells were incubated with 4  $\mu$ M A $\beta$ 1-42 (aged for 24h with heparin), 4  $\mu$ M A $\beta$ 1-42 (aged for 24h with heparin and alginate oligosaccharide derivatives), and alginate oligosaccharide derivatives disassembled A $\beta$ 1-42 (aged for 24h with heparin) for 48h and the cell viability was measured with SRB assay. ## $p < 0.01$  vs control; \*\* $p < 0.01$  vs A $\beta$ 1-42 ( $n = 3$ , means  $\pm$  s.d.)  
A: control B: A $\beta$ +heparin C: A $\beta$ +heparin+alginate oligosaccharide derivatives  
D: alginate oligosaccharide derivatives disassembled A $\beta$ 1-42

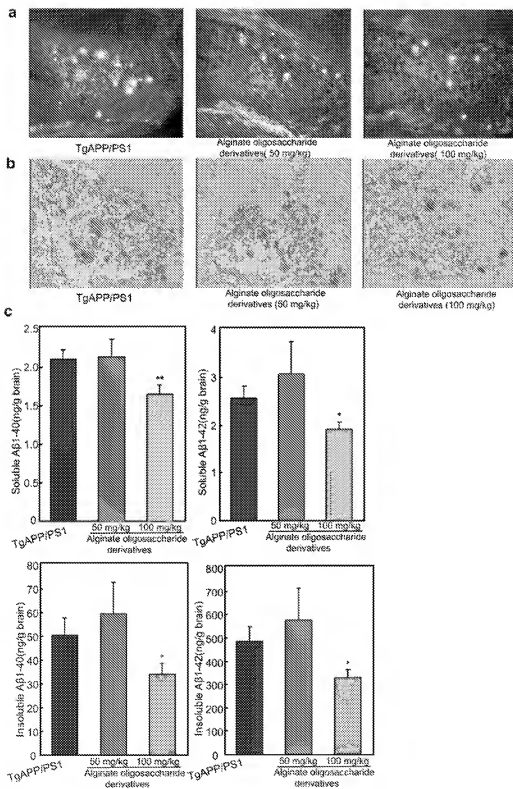
## **Experiment 2**

**Alginate oligosaccharide derivatives, administered before the plaque formation, decrease cerebral A $\beta$  plaque burden and A $\beta$  contents in an APP/PS1 double transgenic mouse model, indicating alginate oligosaccharide derivatives as a both prophylactic and therapeutic drug candidate for AD therapy.**

In APP/PS1 double transgenic mouse model, A $\beta$  peptides start to form plaques at 6 months of age. Alginate oligosaccharide derivatives were orally administered from 5.4 months of age and continued for 8 weeks. Therefore, in this model, we could observe both inhibiting aggregation effect and disassembling effect on A $\beta$ , which reflect the prophylactic effect and therapeutic effect, respectively. The effects of alginate oligosaccharide derivatives on APP/PS1 mouse model were evaluated by cerebral A $\beta$  content and plaque burden.

Senile plaque, an end event of A $\beta$  deposition, was investigated. Thioflavin-S staining showed a statistically significant decrease of 41% ( $P=0.0066$ ) dense plaque load in animals treated with alginate oligosaccharide derivatives (50 mg/kg/day) compared to vehicle control (Fig. 3a). To measure diffuse plaque load, immunohistochemistry using anti-amyloid antibody was applied. A statistically significant decrease (20%,  $P=0.011$ ) in diffuse plaque load was noted in animals treated with alginate oligosaccharide derivatives (50 mg/kg/day) as compared to vehicle control (Fig. 3b).

A $\beta$ 1-40 and A $\beta$ 1-42 contents in both the soluble and insoluble fractions of brain homogenates were measured. ELISAs showed statistically significant decreases of 22% ( $P=0.008$ ) and 26% ( $P=0.021$ ) in soluble A $\beta$ 1-40 and soluble A $\beta$ 1-42, respectively, in animals treated with alginate oligosaccharide derivatives (50 mg/kg/day), compared with vehicle control. Significant decreases in insoluble A $\beta$ 1-40 and A $\beta$ 1-42, of 33% ( $P=0.026$ ) and 31% ( $P=0.016$ ) respectively, were also observed in animals treated with alginate oligosaccharide derivatives (50 mg/kg/day) (Fig. 3c). These results should be attributed to both of the inhibiting aggregation effect and disassembling effect on A $\beta$ .



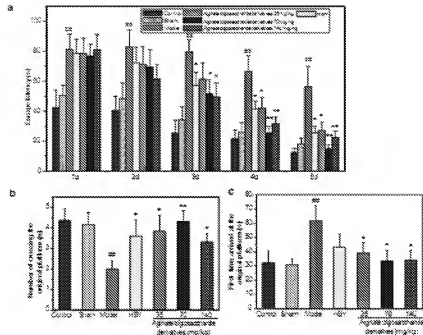
**Figure 3** Effects of alginate oligosaccharide derivatives on A $\beta$  senile plaques and soluble and insoluble A $\beta$  levels in the APP/PS1 double transgenic mouse model. Effects of alginate oligosaccharide derivatives on cerebral A $\beta$  plaques in mouse brain were observed. Brains were fixed and consecutive sections were selected for staining with Th-S for dense plaques (a) or staining with anti-amyloid antibody for diffuse plaques (b). A $\beta$  plaque burdens were assessed with Leica Q-Win software, which was used to convert micrographs to binary images for determination of plaque number and plaque area. (c) Effect of alginate oligosaccharide derivatives on soluble and insoluble A $\beta$ 1-40/A $\beta$ 1-42 in mouse brain. The brains were homogenized followed by Tris/HCl buffer extraction for soluble A $\beta$  or 75% (v/v) trifluoroacetic acid extraction for insoluble A $\beta$ , the levels of which were measured by ELISA assay. \*p < 0.05, \*\*p < 0.01 vs TgAPP/PS1 (n = 12, means  $\pm$  s.e.m.).

### **Experiment 3**

**Alginate oligosaccharide derivatives, preventively attenuate the cognitive dysfunction induced by intracerebroventricular injection of A $\beta$ 1-40 fibril in *Wistar* rats, indicating alginate oligosaccharide derivatives as a prophylactic drug candidate for AD.**

We also employed A $\beta$ 1-40 intracerebroventricular injection model to illustrate the preventive effects of alginate oligosaccharide derivatives. In this model, rats were pretreated with alginate oligosaccharide derivatives for 7 consecutive days before A $\beta$ 1-40 intracerebroventricular injection to observe preventive effects of alginate oligosaccharide derivatives on AD.

Randomly grouped male wistar rats were administered with alginate oligosaccharide derivatives (at doses of 25, 50 and 100 mg/kg) or Huperzine A-treated (HBY, at a dose of 0.2 mg/kg as positive control) orally for 7 consecutive days. On day 8, rats were intracerebroventricularly injected with aged A $\beta$ 1-40, except the vehicle group, which was given sterile water. Following surgery, rats were further treated with drugs or saline. The water maze task was performed on day 19. In acquisition trials, A $\beta$ -treated rats displayed longer escape latency, comparable with the controls. However, this increased escape latency was shortened in a dose-dependent manner by alginate oligosaccharide derivatives (Fig. 4a). In a spatial probe trial, the time arriving at the original platform was shorter in the alginate oligosaccharide derivatives-treated group, compared to the A $\beta$ -treated group (Fig. 4b). In addition, the number of crossing over the original location of the platform was significantly higher in alginate oligosaccharide derivatives-treated group, compared to the A $\beta$ 1-40-treated group (Fig. 4b). HBY, a selective acetylcholinesterase inhibitor, was less potent than alginate oligosaccharide derivatives both in terms of acquisition and spatial probe trials.



**Figure 4** Alginate oligosaccharide derivatives preventively attenuate cognitive dysfunction induced by intracerebroventricular injection of A $\beta$ 1-42 fibril in *Wistar* rats. In Morris water maze test, alginate oligosaccharide derivatives decreased the escape latency (a), decreased the number of original platform crossings and first time arrived at the original platform (b). #p < 0.05 vs control; ##p < 0.01 vs sham; \*p < 0.05, \*\*p < 0.01 vs model (n = 12, means  $\pm$  s.e.m.).

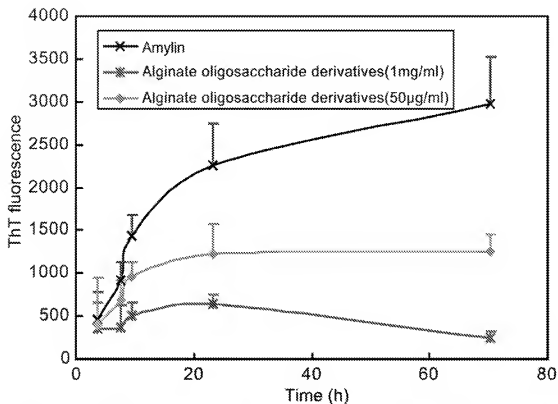
#### Experiment 4

**Alginate oligosaccharide derivatives inhibit the aggregation of amylin and reduce its cytotoxicity to pancreatic beta-cells, promise alginate oligosaccharide derivatives as a prophylactic drug candidate for type 2 diabetes therapy.**

Abnormal aggregation of amylin (islet amyloid polypeptide, IAPP) into amyloid fibrils is a hallmark of type 2 diabetes. Via its amyloidogenic properties and further cytotoxicity, IAPP has been considered to be the key pathogenesis of NIDDM. And amylin aggregation-inhibiting strategy is considered as a both prophylactic and therapeutic treatment for type 2 diabetes.

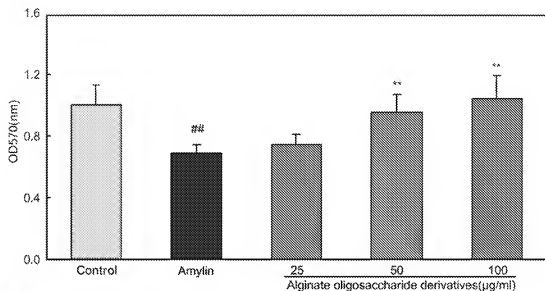
The effects of alginate oligosaccharide derivatives on amylin fibril formation were observed with Thioflavin T (Th-T) assay. Amylin fibril formation was monitored using Th-T fluorescence, a valuable quantitative indicator of  $\beta$ -sheet-rich aggregate, in the absence or presence of alginate oligosaccharide derivatives. In a time course effect, we found that Th-T fluorescence intensity increased gradually in a time-dependent manner (Fig. 4). However, alginate oligosaccharide derivatives (72 h) resulted in a 94.3% decrease in Th-T fluorescence, suggesting that alginate oligosaccharide derivatives, at 1mg/ml, abolish fibril formation of amylin. Notably, alginate oligosaccharide derivatives at 50  $\mu$ g/ml also suppressed, *albeit* less potent, this event.





**Figure 5** Effects of alginate oligosaccharide derivatives on amylin fibril formation as measured by Th-T fluorometric assay.

The protective effects of alginate oligosaccharide derivatives on pancreatic beta-cells impaired by amylin were studied with MTT assay. The pancreatic beta-cells cell line NIT was cultured with DMEM containing 10% FBS. The cells were planted in 96-well plate in density of  $1 \times 10^4$  cells/well. The day after plating, cells were pretreated with varying concentrations of alginate oligosaccharide derivatives for 24 h, followed by the addition of aged amylin with final concentration of 30  $\mu$ M. After 48 h at 37°C, 10  $\mu$ l MTT with concentration of 5 mg/ml were added. After 4 hour at 37°C, the supernatant were removed and 150 $\mu$ l DMSO were added. Then the absorbance at 570nm (630nm as reference) was recorded with an ELISA reader. The results showed that alginate oligosaccharide derivatives could increase the survived cells impaired by amylin in a dose-dependent manner (Fig.5). The data implied alginate oligosaccharide derivatives have the protective effects on cells impaired with amylin.



**Figure 6** Protective effects of alginate oligosaccharide derivatives on NIT cells impaired by amylin. ## $p < 0.01$  vs control, \*\* $p < 0.01$  vs amylin ( $n = 3$ , means  $\pm$  s.d.).

I declare under the penalty of perjury of the laws of the United States of America that the foregoing is true and correct to the best of my information and belief.

Signed by *Meiyu Geng*  
 Print name GENG, Meiyu  
 Date January 14, 2011  
 Place Qingdao